Protein Science (1994), *3*:620–627. Cambridge University Press. Printed in the USA.

Heparin binding domain peptides of antithrombin III: Analysis by isothermal titration calorimetry and circular dichroism spectroscopy

RUTH TYLER-CROSS,[1] MICHAEL SOBEL,[2] DALILA MARQUES,[2] AND ROBERT B. HARRIS[1]

[1] Department of Biochemistry and Molecular Biophysics, Box 614, Virginia Commonwealth University, Richmond, Virginia 23298-0614

[2] Department of Surgery, Division of Vascular Surgery, Box 108, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0108

(RECEIVED December 14, 1993; ACCEPTED January 31, 1994)

Abstract

The serine proteinase inhibitor antithrombin III (ATIII) is a key regulatory protein of intrinsic blood coagulation. ATIII attains its full biological activity only upon binding polysulfated oligosaccharides, such as heparin. A series of synthetic peptides have been prepared based on the proposed heparin binding regions of ATIII and their ability to bind heparin has been assessed by CD spectrometry, by isothermal titration calorimetry, and by the ability of the peptides to compete with ATIII for binding heparin in a factor Xa procoagulant enzyme assay. Peptide F^{123}-G^{148}, which encompasses both the purported high-affinity pentasaccharide binding region and an adjacent, C-terminally directed segment of ATIII, was found to bind heparin with good affinity, but amino-terminal truncations of this sequence, including L^{130}-G^{148} and K^{136}-G^{148} displayed attenuated heparin binding activities. In fact, K^{136}-G^{148} appears to encompass only a low-affinity heparin binding site. In contrast, peptides based solely on the high-affinity binding site (K^{121}-A^{134}) displayed much higher affinities for heparin. By CD spectrometry, these high-affinity peptides are chiefly random coil in nature, but low μM concentrations of heparin induce significant α-helix conformation. K^{121}-A^{134} also effectively competes with ATIII for binding heparin. Thus, through the use of synthetic peptides that encompass part, if not all, of the heparin binding site(s) within ATIII, we have further elucidated the structure-function relations of heparin-ATIII interactions.

Keywords: antithrombin III; binding domains; circular dichroism; heparin; isothermal titration calorimetry; synthetic peptides

Antithrombin III, a plasma glycoprotein which is a member of the serpin superfamily of serine protease inhibitors, inhibits thrombin and factor Xa, as well as several other serine proteases, and thus plays a key regulatory role in intrinsic blood coagulation (Huber & Carrell, 1989; Mourey et al., 1990; Pratt & Church, 1991; Olson & Bjork, 1992). ATIII attains its full inhibitory potential only upon binding polysulfated oligosaccharides, such as heparin, a heterogeneous, sulfated glycosaminoglycan (Casu, 1985).

The interaction of ATIII with heparin is complex and is proposed to occur in stages (Evans et al., 1992). ATIII binds a specific, well-characterized pentasaccharide unit structure within the heparin polymer (Choay et al., 1981; Thunberg et al., 1982; Choay, 1989; Ragazzi et al., 1990), which in turn causes a conformational change in the ATIII molecule (Evans et al., 1992; Olson et al., 1992; Gettins et al., 1993). The high-affinity binding site for the pentasaccharide is thought to be located within the "D" helix of ATIII, the existence of which is predicated on the solved structure of the archetypal serpin, α1-antitrypsin (Huber & Carrell, 1989). The binding of pentasaccharide alone is sufficient to activate ATIII as an inhibitor of factor Xa, but not of thrombin. In order to become an effective thrombin inhibitor, ATIII must bind a longer-chain (up to 14 residues) heparin molecule. The pentasaccharide sequence binds at the high-affinity, D-helix site, and the rest of the heparin chain occupies an adjacent low-affinity binding site that extends in the carboxy-terminal direction toward the active center of ATIII. Occupation of this second site is thought to allow the close approach of thrombin (Evans et al., 1992).

Reprint requests to: Robert B. Harris, Department of Biochemistry and Molecular Biophysics, Box 614, Virginia Commonwealth University, Richmond, Virginia 23298-0614; email: rharris@gems.vcu.edu.

Abbreviations: ATIII, antithrombin III; PNI, protease nexin I; vWF, human von Willebrand factor. Single-letter abbreviations are used to denote amino acids.

Longer-chain heparin molecules (up to 25 residues) are even better at promoting inhibition of thrombin by ATIII. In binding directly to thrombin, these long-chain heparins may function as "conduits" or "guides" that facilitate the formation of the ternary complex between ATIII, heparin, and thrombin (Pomerantz & Owen, 1978; Bjork et al., 1989; Evans et al., 1992; Olson et al., 1992; Ringe, 1992).

Complexation with heparin induces a conformational change in many other proteins, such as fibroblast growth factor (Klagsbrun & Baird, 1991; Prestrelski et al., 1992) or mucous proteinase inhibitor (Faller et al., 1992). The conformational changes exerted upon binding heparin are associated with expression of the full biological activity of the particular protein. Also, work with synthetic peptides has shown that heparin significantly increases the α-helix content and stability of a synthetic 19-residue heparin-binding peptide (Soler-Ferran et al., 1992; Margalit et al., 1993) and induces a conformational change in short-chain synthetic peptides which encompass the heparin binding domain of human von Willebrand factor (Sobel et al., 1992; Tyler-Cross et al., 1993).

We have now prepared a series of synthetic peptides based on proposed heparin binding regions of ATIII and used these peptides to probe and quantify further the binding interaction of ATIII with heparin. The peptides are illustrated in Figure 1: F^{123}-G^{148} encompasses the purported pentasaccharide binding site and an adjacent C-terminally directed region; L^{130}-G^{148} and K^{136}-G^{148} are amino-terminal truncations of F^{123}-G^{148}; and K^{121}-A^{134} and K^{121}-A^{134}Ext are postulated to encompass only the high-affinity, pentasaccharide binding domain and are in effect carboxy-terminal truncations of F^{123}-G^{148}. All peptides were tested for their ability to bind heparin as assessed by isothermal titration calorimetry, CD spectrometry, and a competitive binding assay.

Titration calorimetry data show that all the peptides possess some affinity for heparin, but those that encompass the high-affinity pentasaccharide site bind heparin with the highest affinity. Similarly, only the high-affinity site peptides undergo alterations in secondary structure with a significant increase in α-helix content upon complexation with heparin. Peptides encompassing the high-affinity site were also effective competitors of ATIII for binding heparin. This investigative approach affords a direct, quantitative assessment of the interaction of heparin and potential heparin binding peptides.

Results

Design of the high-affinity heparin binding site peptide of ATIII

In ATIII, the high-affinity pentasaccharide binding site is thought to reside primarily on the D helix, approximately within the segment F^{123}-A^{134} (Fig. 1). In the native protein, the positive charge contributed by Arg^{47} (resident on the A helix) is also postulated to be an essential component of the high-affinity site because in the folded protein, Arg^{47} is likely brought into close spatial proximity to the D helix (Evans et al., 1992). Similarly, the high-affinity pentasaccharide binding site of protease nexin I (PNI) also resides on a helix, but in this case, the positive charge provided by Arg^{47} at the ATIII site is supplied by Lys^{121} (Evans et al., 1992). In order to construct a contiguous peptide that mimics the high-affinity binding site of ATIII, F^{123}-A^{134} was thus synthesized but in addition, an N-terminal extension, Lys-(β-Ala), was added (Fig. 1). This puts a Lys residue in a position equivalent to what would be Lys^{121} in PNI, theoretically mimicking the positive charge contributed by Arg^{47} in ATIII. This peptide is designated K^{121}-A^{134}. β-Ala was included to allow flexibility in the N-terminal region of the peptide, thereby increasing the chances for proper alignment of the Lys residue for productive electrostatic interaction with heparin. A second peptide was prepared, K^{121}-A^{134}Ext (Fig. 1) in which this core sequence was "extended" at the N-terminus by a sequence motif known to promote α-helix formation (Soler-Ferran et al., 1992). In both of these peptides, Ala was substituted for N^{127} and C^{128} in order to favor helix formation and to preclude the possibility of disulfide bond formation.

Isothermal titration calorimetry

Isothermal titration calorimetry was used to directly assess binding between the ATIII peptides and heparin. A representative

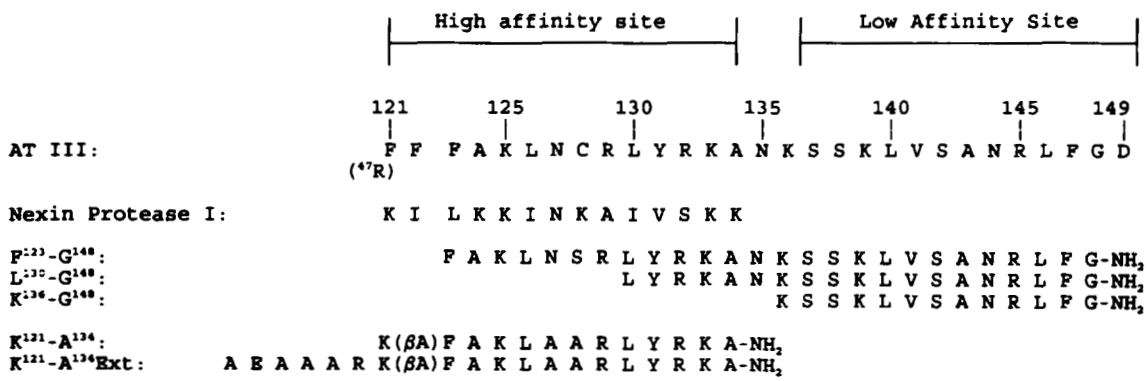

Fig. 1. Potential heparin binding domain peptides of ATIII. The high-affinity pentasaccharide binding site of ATIII resides on the D helix approximately within residues F^{121}-A^{134} and is adjacent to a purported low-affinity heparin binding site (6). Within the folded protein, R^{47}, on the A helix, is thought to contribute an essential cationic charge to the high-affinity binding site. In protease nexin I, the high-affinity helix segment contains K^{121} at its N-terminus. Hence, peptides were synthesized so as to potentially encompass only the low-affinity site (K^{136}-G^{148}), portions of both binding sites (F^{123}-G^{148}; L^{130}-G^{148}), or only the high-affinity site (K^{121}-A^{134}; K^{121}-A^{124}Ext).

binding isotherm obtained with the F^{123}-G^{148} peptide at 25 °C demonstrates that binding with heparin is exothermic and saturable (Fig. 2). The relevant thermodynamic parameters were determined by deconvolution of the binding isotherm, and titration calorimetry experiments were done for all other peptides in this study (Table 1). All experiments were done at least in triplicate.

As shown, peptides K^{121}-A^{134} and K^{121}-A^{134}Ext possess the highest affinities for heparin (K_D's in the range of 1×10^{-8} M; Table 1). These results substantiate our peptide design scheme in that these peptides clearly encompass a high-affinity heparin binding site.

Peptide K^{136}-G^{148}, which contains none of the residues ostensibly involved in the pentasaccharide binding domain, showed the lowest affinity for heparin (Table 1). However, this peptide clearly retains some affinity for heparin ($K_D = 1.1 \pm 0.1 \times 10^{-5}$ M), possibly because this sequence encompasses a part of the low-affinity heparin binding site within ATIII.

Somewhat surprisingly, F^{123}-G^{148}, which is theorized to contain the majority of the high-affinity site plus the adjacent low-affinity site sequence, binds heparin with poorer affinity than either K^{121}-A^{134} or K^{121}-A^{134}Ext. In fact, L^{130}-G^{148}, which contains only a small portion of the high-affinity site plus the adjacent low-affinity site sequence, has a slightly higher affinity for heparin than F^{123}-G^{148}. Previous control experiments showed that peptides that do not bind heparin also do not yield a titration binding isotherm (Tyler-Cross et al., 1993).

The anomalous results obtained by titration calorimetry with the F^{123}-G^{148} peptide may be due to the inherent limitations of mathematical curve fitting. The assumption of a single binding site for this peptide consistently resulted in relatively large standard errors of fit, which results in the relatively large standard deviation about the mean of the calculated thermodynamic parameters (Table 1). At low concentrations of heparin, the data points for this peptide form a characteristic convex deviation from the curve. In contrast, the data obtained with K^{121}-A^{134}, K^{121}-A^{134}Ext, or K^{136}-G^{148} resulted in very small standard errors of fit for the 1-site binding model. It is possible, therefore, that the association constant calculated for the F^{123}-G^{148} peptide is artifactually low. Fitting the binding data obtained with the F^{123}-G^{148} peptide to a 2-binding site model resulted in small standard errors of fit, which allowed calculation of 2 association constants; $K_{D_{\text{high affinity}}} = 3.5 \times 10^{-9}$ M; $K_{D_{\text{low affinity}}} = 3.5 \times 10^{-5}$ M. Thus, the F^{123}-G^{148} peptide appears to encompass 1 high- and 1 low-affinity heparin binding site. Although these results are consistent with the presence of 2 sites, it should be pointed out that mathematical deconvolutions of this type cannot unambiguously determine the true number of different affinity sites.

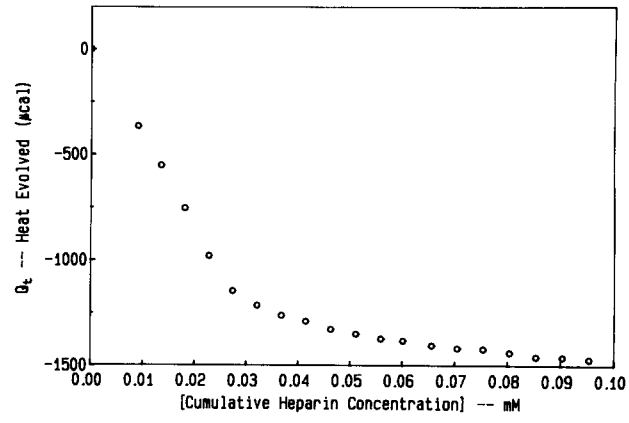

Fig. 2. Representative binding isotherm obtained by isothermal titration calorimetry of the F^{123}-G^{148} peptide. This experiment was done at 25 °C; the peptide (0.250 mM in the calorimeter cell) and heparin (0.625 mM in the dropping syringe) were dissolved in 30 mM phosphate buffer, pH 7.0.

For several of the peptides ΔCp values were calculated from the enthalpy terms by performing the titration experiments at 2 different temperatures. In all cases, for the interaction of heparin and the peptides, ΔCp was negative, but of relatively small magnitude (e.g., for F^{123}-G^{148}, $\Delta Cp = -0.900$ kcal/mol and for K^{121}-A^{134}Ext $\Delta Cp = -2.360$ kcal/mol). This suggests that, while some hydrophobic bonds are formed upon heparin binding, the majority of the binding energy is not due to hydrophobic interactions.

Far UV CD measurements

We (Sobel et al., 1992; Soler-Ferran et al., 1992; Tyler-Cross et al., 1993) and others (Evans et al., 1992; Lellouch & Lansbury, 1992) have used CD spectrometry to monitor protein or peptide conformational changes that accompany complex formation with heparin. CD was therefore used to study the interaction of heparin with the ATIII-based peptides.

The addition of incremental amounts of heparin to a solution of the F^{123}-G^{148} peptide results in a concentration-dependent reorganization of the peptide structure (Fig. 3), which is clearly indicated by the change in the shape of the CD spectral envelope and the concomitant displacement of the absorption maxima

Table 1. *Thermodynamics of heparin binding by the ATIII peptides*[a]

Peptide	K_D (M)	ΔH (kcal/mol)	ΔS (eu)	N	$1/N$
F^{123}-G^{148}	$9.3 \pm 6.6 \times 10^{-7}$	-40.5 ± 3.8	-121 ± 14	0.135	7 ± 0.5
L^{130}-G^{148}	$3.5 \pm 0.6 \times 10^{-7}$	-42.0 ± 0.0	-125 ± 1.2	0.097	10 ± 2.4
K^{136}-G^{148}	$1.1 \pm 0.1 \times 10^{-5}$	-39.7 ± 4.0	-121 ± 15	0.088	11 ± 1.4
K^{121}-A^{134}	$8.1 \pm 2.7 \times 10^{-9}$	-76.8 ± 1.7	-244 ± 1.7	0.034	29 ± 0.8
K^{121}-A^{134}Ext	$1.8 \pm 0.9 \times 10^{-8}$	-67.3 ± 1.7	-210 ± 8.1	0.097	10 ± 1.6

[a] All experiments were done at 30 °C; ±1 SD.

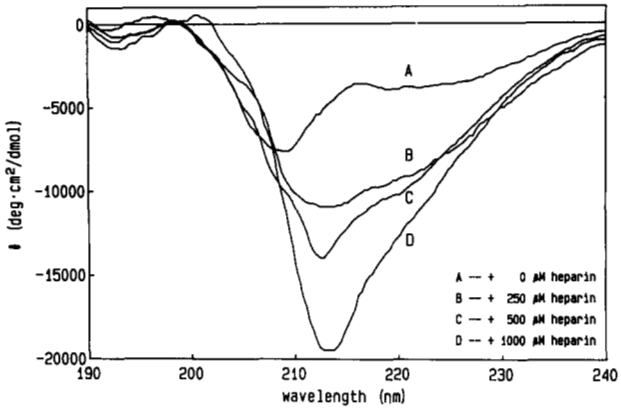

Fig. 3. Far UV spectra of the F^{123}-G^{148} peptide alone and in the presence of increasing concentrations of heparin. The spectra were acquired using 100 μM peptide in 30 mM phosphate buffer, pH 7.0. Increasing the heparin concentration caused a pronounced, reproducible shift in the spectral envelope and in the position of the absorption maxima and minima.

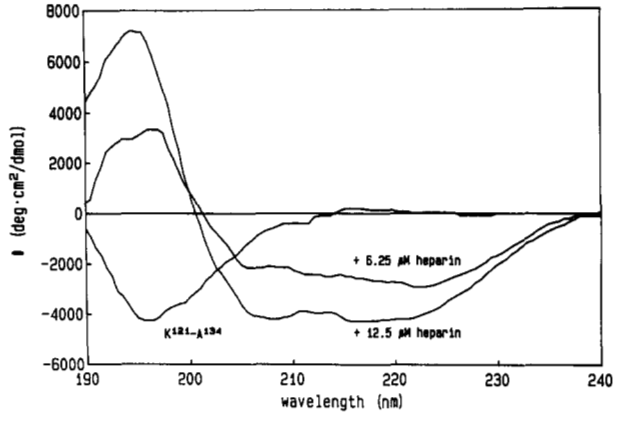

Fig. 4. Far UV spectra of the K^{121}-A^{134} peptide alone and in the presence of increasing concentrations of heparin. As little as 6.25 μM heparin induced α-helix formation in this peptide.

and minima. The CD spectrum obtained for the peptide–heparin complex cannot be accounted for by the simple superposition of the individual spectra obtained for the peptide alone plus heparin alone; the spectrum for the complex is already corrected by subtraction of the spectrum obtained for the appropriate concentration of heparin. Furthermore, peptides that do not bind heparin do not undergo a conformational change observable in the CD upon addition of heparin (Tyler-Cross et al., 1993).

These results are essentially consistent with those of Lellouch and Lansbury (1992) who showed by CD that heparin induces secondary structural changes in the ATIII-based peptide, F^{123}-K^{139}. However, in the work presented here, much lower concentrations of heparin were used because we found that virtually all peptides tested showed some degree of alteration in spectral envelope at high (>1 mM) heparin concentration.

The N-terminal truncated peptides L^{130}-G^{148} and K^{136}-G^{148} were also analyzed by CD. Neither peptide showed a discernible change in its spectral envelope except at very high (>500 μM) concentrations of heparin (data not shown). Although these peptides bind heparin (as shown by titration calorimetry), heparin binding does not appear to significantly alter their conformation.

Although the segment K^{121}-A^{134} of ATIII is postulated to be part of an α-helix in the native protein, the synthetic peptide K^{121}-A^{134} possesses no α-helix character at neutral pH (Fig. 4), regardless of the concentration (up to 200 μM) or temperature (4 or 25 °C) tested. This peptide was purposely synthesized to contain Ala residues in place of Asn^{127} and Cys^{128} (Ala residues are known to promote helix formation [see Soler-Ferran et al., 1992]), yet this peptide lacks helix character. Likewise, K^{121}-A^{134} is devoid of α-helix character in borate buffer (pH 10) or in 70% (v/v) ethanol solution (data not shown). However, as little as 6.25 μM heparin causes a dramatic secondary structural rearrangement of the peptide, resulting in a small, but reproducible increase in α-helix content (Fig. 4). Maximum helix formation occurs in 12.5 μM heparin; addition of up to 100 μM heparin does not cause any further changes in the spectral envelope (Table 2). At 12.5 μM heparin, the peptide is about 6% α-helix.

In a further attempt to induce helix formation in K^{121}-A^{134} in the absence of heparin, the peptide was synthesized with an amino-terminal extension (A-E-A-A-A-R-) previously shown to facilitate helix formation (Soler-Ferran et al., 1992). The resulting peptide, K^{121}-A^{134}Ext, however, also displays little tendency toward helix formation at 4 or 25 °C, or at pH 7.0 or 10.0. In 70% (v/v) ethanol, the peptide assumes 6% helix character (data not shown). As noted with the K^{121}-A^{134} peptide, however, heparin binding to K^{121}-A^{134}Ext causes a reorganization in structure with a significant increase in α-helix content (Table 2). In fact, the K^{121}-A^{134}Ext peptide assumes greater helix character in 12.5 μM heparin (15%) than does the K^{121}-A^{134} peptide. As with the K^{121}-A^{134} peptide, maximum helix formation was observed at 12.5 μM heparin; addition of up to 100 μM heparin caused no further change in the spectral envelope.

Upon addition of heparin to the K^{121}-A^{134}Ext peptide, a fine precipitate forms in the solution. This is not unusual and sometimes occurs with other high-affinity heparin binding peptides (Gelman & Blackwell, 1973; Tyler-Cross, unpubl. obs.). The

Table 2. *Effect of heparin on the distribution of secondary structural elements of the K^{121}-A^{134} peptide and K^{121}-A^{134}Ext peptide*[a]

	Fractional % distribution			
Heparin (μM)	α-Helix	β-Strand	β-Turn	Unordered
	K^{121}-A^{134} peptide			
0	0.00	65.0	9.37	25.5
6.25	3.71	75.5	3.42	17.3
12.5	6.25	76.5	0.00	17.2
	K^{121}-A^{134}Ext peptide			
0	0.00	63.3	6.73	29.9
6.25	11.1	51.7	13.2	38.3
12.5	14.9	39.0	12.9	33.1

[a] Spectra were obtained in 30 mM phosphate buffer, pH 7.0, and each was corrected by subtraction for the spectrum obtained with heparin alone at the indicated concentration.

precipitate is likely due to the creation of a lattice network of the peptide-heparin complex. Light scattering due to the particulate nature of the solution may interfere somewhat with the CD experiment, but nonetheless, it is clear that a marked shift from unordered to ordered structure occurs upon the addition of small amounts of heparin to the peptide solution. The increase in helix content comes at the expense of other secondary structural features (Table 2).

Competitive binding assay results

As expected (Fig. 5), F^{123}-G^{148}, L^{130}-G^{148}, K^{121}-A^{134}, and K^{121}-A^{134}Ext, are all potent competitors of ATIII for binding heparin with IC_{50}'s ranging from 0.3–0.7 μM. Note that with each of these peptides, nearly 100% inhibition could be obtained at greater than 5 μM peptide. In contrast, even at high concentration, the K^{136}-G^{148} peptide achieves only 55% maximal inhibition.

If the K^{136}-G^{148} peptide contains only the low-affinity binding site and the K^{121}-A^{134} peptides contain chiefly the high-affinity site, then each peptide is likely binding a different unit structure or site within heparin. If the heparin sites do not overlap, then each peptide might bind independently, and combinations of the peptides assayed together should show a greater inhibitory effect than either peptide assayed alone. On the other hand, if the heparin unit structures overlap, the K^{121}-A^{134} peptides might be expected to completely exclude binding by the K^{136}-G^{148} peptide because the K^{121}-A^{134} peptides bind heparin with much greater affinities than the K^{136}-G^{148} peptide and the observed inhibition should correlate solely with the concentration of the K^{121}-A^{134} peptide.

This experiment was done by studying the ability of mixtures of the peptides to compete with ATIII for binding heparin. Thus, K^{136}-G^{148} plus K^{121}-A^{134} or K^{136}-G^{148} plus K^{121}-A^{134}Ext were incubated at their respective IC_{50} concentrations, and the inhibition of ATIII-heparin binding was assessed by competitive binding assay. As shown (Fig. 6), the ability of K^{136}-G^{148} plus K^{121}-A^{134} or K^{136}-G^{148} plus K^{121}-A^{134}Ext to inhibit ATIII-heparin binding is essentially equal to the sum of the percent inhibition obtained for each peptide alone (Fig. 6B). In other words, K^{136}-Gly^{148} does not compete with either of the K^{121}-A^{134} peptides for binding heparin and together, the high- and low-affinity site peptides compete more effectively with native ATIII for binding heparin.

Doubling the K^{136}-G^{148} concentration (Fig. 6B) did not change the percent inhibition of heparin binding to factor Xa, but doubling the concentration of either of the K^{121}-A^{134} peptides or mixing the 2 K^{121}-A^{134} peptides together (Fig. 6C) increased the percent inhibition of heparin binding to the values predicted by the curves in Figure 5.

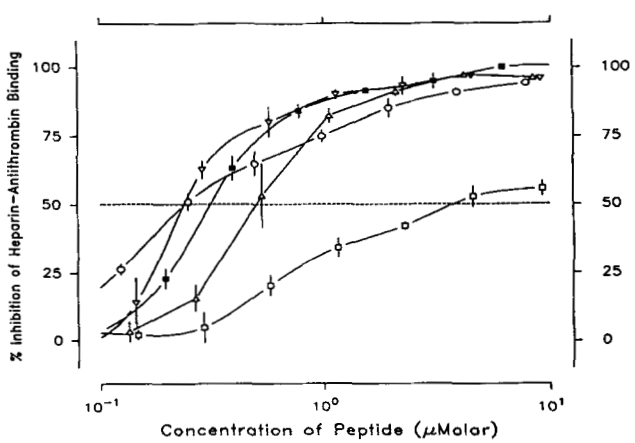

Fig. 5. The inhibitory effects of synthetic antithrombin peptides on heparin-ATIII complex formation. Using the competitive binding assay, increasing concentrations of each peptide were incubated with unfractionated heparin and ATIII. The resulting heparin-ATIII complex formation was measured by neutralization of Factor Xa enzyme activity. ■, F^{123}-G^{148}; ▽, K^{121}-A^{134}Ext; △, K^{121}-A^{134}; ○, L^{130}-G^{148}; □, K^{136}-G^{148}. In the absence of any of the peptides, Factor Xa is inhibited nearly 90% relative to its activity in the absence of heparin or ATIII. The results are expressed as the percent inhibition of heparin-induced suppression of Factor Xa activity.

Discussion

In the absence of crystal structures of ATIII-heparin complexes, indirect methods are being used to determine the locus of the heparin binding site(s). To date, inferential information has been obtained from many diverse methodological studies (Smith & Knauer, 1987; Smith et al., 1990; Sun & Chang, 1990; Pratt & Church, 1991) and α1-antitrypsin, the prototypical serpin, has been crystallized in its reacted (cleaved) form with bound heparin (Lobermann et al., 1984). These findings lead to a working hypothesis for the mechanism(s) by which heparin activates ATIII (Evans et al., 1992). The minimal heparin pentasaccharide unit structure that binds to ATIII at the high-affinity site has been determined (Choay et al., 1981; Choay, 1989; Ragazzi et al., 1990; Olson et al., 1992). This high-affinity site is thought to be discontinuous, encompassing residues 121–133 of the D helix and residues in the amino-terminal region of the protein, particularly Arg^{47}, resident on the A helix. A low-affinity heparin binding site is postulated to reside on the carboxy-terminal side of the pentasaccharide site, and residues through at least Arg^{145} are likely exposed at the surface of the protein (Smith et al., 1990; Sun & Chang, 1990), lending credence to the idea that they constitute a potential binding site. The results presented here are entirely consistent with the existence of high- and low-affinity heparin binding sites within ATIII. Furthermore, synthetic peptides based on the implicated sequences retain their expected rank order of heparin binding affinities.

Characteristics of binding by the synthetic peptides

We have used isothermal titration calorimetry to show that K^{121}-A^{134} and K^{121}-A^{134}Ext, which were designed to encompass only the high-affinity site, possess K_D values about 1,000-fold lower than K^{136}-G^{148}, which is postulated to contain only a low-affinity heparin binding site. When the binding data obtained with F^{123}-G^{148} (which includes both sites) were fitted to a 2-site binding equation, a high- and a low-affinity association constant were determined. These constants are in very good agreement with the values obtained from the individual peptides that are postulated to contain exclusively the high- (the K^{121}-A^{134} peptides) and low- (K^{136}-L^{148}) affinity sites. Heparin is a polydisperse material that probably contains numerous chemically unique unit structures possessing defined biological activities. Hence, the heparin unit structure that binds at the

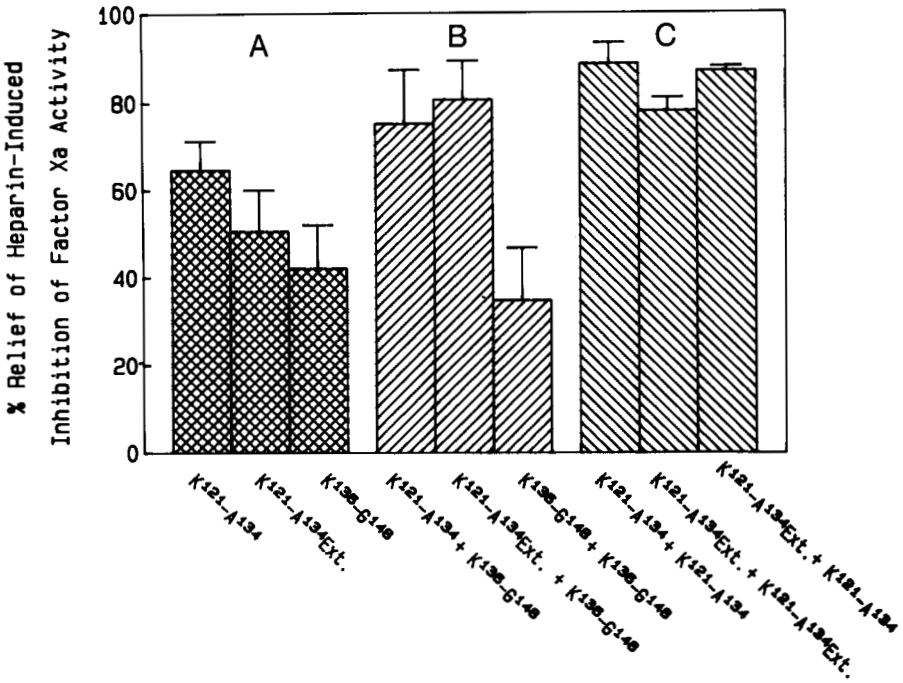

Fig. 6. Additive effects of the synthetic peptides on their ability to relieve inhibition of Factor Xa activity induced by heparin–ATIII. **A:** K^{121}-A^{134} (0.51 μM), K^{121}-A^{134}Ext (0.22 μM), and K^{136}-G^{148} (2.0 μM) were tested individually for their ability to relieve inhibition of Factor Xa activity. **B:** Each peptide was then incubated with an additional K^{136}-G^{148} (2.0 μM) and Factor Xa activity was redetermined. Here, the concentration of K^{136}-G^{148} is effectively doubled over the concentration used in A. **C:** The concentrations of K^{121}-A^{134} or K^{121}-A^{134}Ext were effectively doubled over the concentrations used in A or equivalent concentrations of K^{121}-A^{134} plus K^{121}-A^{134}Ext were incubated together. Again, restoration of Factor Xa activity was determined. Each experiment was done at least 3 times; error bars are indicated.

low-affinity site may well be of a different chemical structure than the pentasaccharide that binds at the high-affinity site. For all intents and purposes, however, heparin behaves as a homogeneous ligand in these and other binding assays (Sobel & Adelman, 1988).

The rank order of the different peptide binding affinities for heparin was similar, whether estimated calorimetrically or by competitive binding assay. However, the K_D's as estimated from titration calorimetry were approximately an order of magnitude lower than the IC_{50}'s derived from the ATIII–heparin binding assay. The 2 methods may reflect different stoichiometries and binding interactions. That is, in the competitive binding assay, only peptides bound to the ATIII-specific pentasaccharide of heparin will demonstrate inhibition; peptide binding to other nonessential regions of heparin would not be observed. Conversely, peptide-heparin binding events may be observed in titration calorimetry whether or not the pentasaccharide is involved. This emphasizes the importance of the next phase of investigation, namely, to analyze the interactions between the peptides and structurally homogeneous glycosaminoglycans.

In order to subfractionate crude heparin into species that may possess different binding affinities, the ATIII peptides are now being immobilized as potential affinity ligands following a recently elaborated strategy (Tyler-Cross et al., 1993).

Complex formation between the peptides and heparin is thermodynamically favored (overall negative G) by large negative enthalpy and relatively small entropy changes. Binding is overwhelmingly enthalpically driven. This finding is typical for the interaction of proteins with carbohydrate ligands (Imberty et al., 1991) and is usually ascribed to changes that occur in the solvation state of the ligand and/or macromolecule mediated by the formation of hydrogen bonds or hydrophobic, electrostatic, or dipole-dipole interactions that occur at the macromolecule-ligand interface (Eftink & Biltonen, 1980).

Heparin binding by proteins and peptides is thought to be dependent primarily on the formation of productive electrostatic interactions between sulfate and carboxyl groups of heparin and suitably spaced cationic residues of the peptide or protein (Cardin & Weintraub, 1989; Sobel et al., 1992; Soler-Ferran et al., 1992; Margalit et al., 1993; J.B. McCarthy & L.T. Furcht, June 13, 1989, US Patent 4,839,464). While some hydrophobic interactions probably occur upon complex formation, these forces clearly do not play a major role in mediating binding between heparin and the ATIII-based peptides. The ΔCp for the interaction between the peptides and heparin was of relatively small magnitude compared with the enthalphic term.

Stoichiometry of binding

In all cases, the calculated stoichiometry, N, was less than unity. A more useful parameter is $1/N$, which we interpret to mean the number of peptide equivalents bound per "average" heparin monomer (15,000 M_r). For all the ATIII peptides, $1/N > 1$, suggesting that there are multiple binding sites for each peptide along the heparin backbone. In general, a rough inverse correlation exists between peptide chain length and the number of equivalents bound per heparin monomer in that the longer the peptide, the fewer equivalents are bound. However, this correlation does not hold true for the high-affinity peptides, K^{121}-A^{134}Ext (20 residues, $1/N = 10$) and K^{121}-A^{134} (14 residues, $1/N = 30$) where binding is dominated by exceedingly large enthalpic terms. K^{121}-A^{134} appears to bind heparin at an unusually high density when compared to the other peptides.

We reported similar results for peptides based on vWF, which binds heparin (Tyler-Cross et al., 1993), and recently the same type results were found for binding between acidic fibroblast growth factor and heparin (Mach et al., 1993). In that case, ap-

proximately 14–15 molecules of growth factor were found to bind per 16,000 molecular weight heparin "monomer."

Effect of heparin on peptide conformation

Only those peptides that encompass the putative high-affinity binding site display an alteration in secondary structure upon binding heparin. Changes in secondary structure alone as monitored by CD do not formally prove ligand binding but we (Sobel et al., 1992; Tyler-Cross et al., 1993; You et al., 1993) and others (Lellouch & Lansbury, 1992) have shown that in some cases heparin binding induces conformational changes that are observable in the CD. Heparin has previously been shown to stabilize protein and peptide conformers in solution and to impart thermal and chemical stability to peptide–heparin complexes (Evans et al., 1992; Faller et al., 1992; Olson et al., 1992; Prestrelski et al., 1992; Sobel et al., 1992; Soler-Ferran et al., 1992). Maximum changes in secondary structure of the K^{121}-A^{134} peptides were observed at μM concentrations of heparin, whereas with the F^{123}-K^{139} peptide, mM concentrations were needed to induce maximum changes in structure (Lellouch & Lansbury, 1992). Clearly, there is a marked difference in the heparin binding affinity of the various ATIII peptides.

Although K^{121}-A^{134} and K^{121}-A^{134}Ext possess no helix character in the absence of heparin, binding induces the assumption of some helix structure. This result is similar to our previous findings with specifically designed helix peptides where heparin binding significantly increased the fractional percentage of helix structural elements (Soler-Ferran et al., 1992). Although this segment of ATIII is postulated to exist in a helix in the native protein, there is currently no direct experimental evidence to support this supposition. To our knowledge, this is the first demonstration that this sequence is capable of assuming helix structure (albeit a very small fractional percentage of helix structure) upon binding low concentrations of heparin.

We can now speculate that high- and low-affinity heparin unit structures exist that are chemically distinct and that must lie in separate regions of the heparin chain. Peptides K^{136}-G^{148}, K^{121}-A^{134}, and K^{121}-A^{134}Ext are each potent inhibitors of ATIII for binding heparin. In contrast, regardless of its concentration, K^{136}-L^{148} does not cause 100% inhibition of ATIII–heparin binding. When incubated together with the high-affinity peptides, the percent inhibition of ATIII–heparin binding is essentially equal to the sum of the effects obtained with each peptide alone. Hence, K^{136}-L^{148} and K^{121}-A^{134} or K^{121}-A^{134}Ext can bind to heparin simultaneously, suggesting that their respective heparin binding sites do not overlap. Because the effects of the high- and low-affinity site peptides are additive, we can conclude that binding both the high- and low-affinity heparin structures are necessary for full potentiation of biological activity.

Furthermore, the ATIII peptides are much less potent competitors of the vWF peptides in the vWF/platelet/heparin assay system (Sobel et al., unpubl. obs.). It is likely, then, that the heparin structure that binds to the K^{121}-A^{134} ATIII peptides is chemically different than the heparin structure that binds to vWF (Tyler-Cross et al., 1993). This result is consistent with the hypothesis that the various biological activities of heparin are associated with distinct chemical structures. We are working toward detailing the chemical make-up of the high-affinity heparins.

Materials and methods

Peptide synthesis

All peptides were synthesized by solid-phase methods (Milligen model 9600 Peptide Synthesizer), purified to N-terminal homogeneity, and analyzed and characterized as previously described (Sobel et al., 1992; Soler-Ferran et al., 1992; Tyler-Cross et al., 1993).

Isothermal titration calorimetry

Isothermal titration calorimetry was used to directly assess binding between the ATIII peptides and heparin. All experiments were carried out using an Omega titration calorimeter (Microcal, Inc.) essentially as described previously (Tyler-Cross et al., 1993; You et al., 1993). In a typical experiment, 15 or 20 12-μL aliquots of heparin were injected into 1.396 mL of peptide solution with rapid mixing at 400 rpm; there were 3-min intervals between injections. Heats of reaction were determined by integration of the observed peaks. The experimental temperature was 30 °C unless otherwise indicated, and the buffer was 30 mM phosphate, pH 7.0. No baseline correction for heat of mixing was necessary based on the results of control experiments in which aliquots of heparin were injected directly into buffer. The reference cell of the calorimeter contained water plus 0.01% sodium azide. The cumulative total of the heat evolved was plotted against the total ligand concentration to produce a binding isotherm. By curve-fitting the data to an equation that describes ligand binding to a macromolecule possessing 1 set of independent ligand sites, the following thermodynamic parameters were obtained: the association constant, K_A (M); the enthalpy change, ΔH (kcal/mol); the entropy change, ΔS (cal/mol·K; entropy units, eu); and N, the stoichiometry of ligand molecules (heparin) bound per equivalent of peptide. The data were deconvoluted using the nonlinear least squares algorithm supplied in the software of the manufacturer. The average unit molecular weight of heparin was estimated to be 15,000.

CD spectrometry

CD spectra were obtained with a Jasco J-500C spectropolarimeter scanning from 260 to 190 nm at a scan rate of 10 nm/min. The temperature of the solution cell was maintained at ±0.1 °C with a circulating water bath. A 1-mm-pathlength optical cell was used. The observed ellipticity was normalized to units of $deg \cdot cm^2 \cdot dmol^{-1}$, and the precise peptide concentration was determined by amino acid compositional analysis of the stock solutions from which the experimental samples were diluted. All dichroic spectra were smoothed and corrected by background subtraction for the spectrum obtained with buffer alone or for buffer containing the indicated concentration of heparin.

The spectra in the region 240–190 nm were analyzed in order to determine the fractional percent of secondary structural elements (Chang et al., 1978) using the PROtein SECondary structure program. This program calculates percent α-helix, β-strand, and β-turn elements, and all remaining structure are considered to be unordered or random coil. The fractional percentage of each structural element, f, must be in the range $0 \leq f \leq 1$, and the sum total of all fractional elements must equal 1. In some instances, the fractional percentage α-helix was also calculated

by the method of Chakrabartty, as previously described (Soler-Ferran et al., 1992).

Competitive binding assay

To measure the ability of the synthetic peptides to compete with native ATIII binding to unfractionated heparin, we adapted the heparin assay devised by Teien and Lie (1977). This assay measures heparin-antithrombin complex formation by its neutralization of activated Factor X (Xa). The reagents were supplied by Kabi (Stockholm). Heparin (28 nM), human ATIII (280 nM), and the synthetic peptide (0–10 μM) were co-incubated at room temperature in the wells of a microtiter plate for 15 min. The ATIII preparation was judged pure on the basis of SDS-PAGE, which revealed a single band on Coomassie blue staining. Subsequently, Factor Xa and then a chromogenic substrate for Factor Xa were sequentially added, and the residual activity of Factor Xa was measured colorimetrically. Peptide binding to the antithrombin domain of heparin diminished the formation of heparin-antithrombin complex, and more residual Xa activity was consequently observed. The degree of inhibition caused by the peptide was calculated as the percent reduction of heparin-antithrombin complex activity in the absence of peptide.

Acknowledgments

This work was supported in part by USPHS grant HL 39903 (M.S.), by grants from Glycomed, Inc., Alameda, California, to R.B.H. and M.S., and by a grant from Commonwealth Biotechnologies, Inc. to R.B.H.

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